# Chapter 8: Trimetallic mixture toxicity and protective effect of melatonin: *In Vivo* structural and functional alterations of testis and epididymis and epididymal sperms and *In Vitro* alterations in cell viability and testosterone secretion

## Introduction:

Sexual reproduction is achieved essentially by the participation of both male and female gametes and, the event of formation of gametes is referred to as gametogenesis. Male gametogenesis is designated as spermatogenesis i.e. formation of sperm (male gamete). Starting from its journey as spermatogonia till it attains its total functional ability to fertilize egg, sperm undergoes two main events, to name them, spermatogenesis (including spermiation) and sperm maturation. Interestingly, both these events directly or indirectly are dependent on androgen that is produced in the testis by the process of steroidogenesis (Fujii *et al.*, 2003). Though the sperms are produced in the testis, they attain their functional competence (sperm maturation) in the epididymis (Vernet *et al.*, 2004). Thus the roles of both these male reproductive organs are indispensable for the formation of mature sperm having ability to fertilize ova, leading to the conclusion that, any disturbances due to any exogenous or endogenous factors, in these two male reproductive organs, may lead to low quantity and quality of sperms increasing the risk of male infertility. Further, testis and sperms present in the epididymis are highly vulnerable to oxidative stress (OS) due to their high content of poly unsaturated fatty acids (PUFA). Hence, it is of great concern that, both these organs should maintain a proper equilibrium between pro-oxidants and antioxidants. Data of the previous chapters (Chapter 2 - 6) have suggested derailment

of antioxidant defense and increased lipid peroxidation under metal exposure either alone or in combination.

Though, there are many studies available on the effects of exposure to individual metals, there are hardly two or three studies available related to multiple metal exposure and male reproductive system. As a matter of fact, studies related to combination metal exposure are more relevant to humans, as humans are never exposed to an individual metal in the environment; instead, always are exposed to a combination of metals. Hence, the studies focusing on multiple metal induced changes in any organ system, including male reproductive system and that too for a longer duration of exposure with a realistic dosage, is more relevant than extrapolating the results obtained from the usage of standard toxicological doses based on $LD_{50}$, NOEL, LOEL, etc. as was in vogue in most of such studies (Benoff *et al.*, 2008). Moreover, the routes of exposure (intraperitoneal, intradermal, intramuscular, intravenous, and subcutaneous) employed in many of the previously cited investigations are not the natural mode of entry of xenobiotic compounds, including metals, into the human body.

Previous study from our laboratory had suggested high contents of chromium, cadmium and nickel in the dietary sources of Vadodara population (Sharma, 1996). Toxicity due to oxidative stress induced by these three metals have been clearly elucidated (Chapters 1, 2, 3) and has also been documented in literature (Valko *et al.*, 2005). Since there was scarcity of data on metallic mixture induced toxicity of male reproductive system, the effect of a mixture of Cd, Cr and Ni in induction of oxidative stress in testis and epididymis of *Wistar* rats was studied (Chapter - 4). The findings from the above study suggested increased lipid peroxidation, low levels of non-

enzymatic antioxidants and inhibition in the activity of antioxidant enzymes as the features.

In continuation, the present study intends to evaluate further, toxicity manifestations on exposure to the metallic mixture in terms of spermatogenesis, steroidogenesis, sperm maturation, activity of steroids dehydrogenases enzymes (3β HSD and 17β HSD), serum titres of testosterone and estradiol and sperm parameters (epididymal sperm count, motility and abnormality), in a temporal sequence of exposure to the metallic mixture. Further, to confirm the results of *In Vivo* findings, a primary culture of isolated Leydig cells was challenged to the metal mixture under *In Vitro* conditions in terms of cell viability and, testosterone production under basal and hCG stimulated conditions. Interestingly, this would be the first investigation of its kind in which the effects of natural route of exposure (oral route) of a realistic dosage of metal combination on serum NO levels and apoptosis in testis (TUNEL assay and Caspase3 activity) are being explored.

It is said that "prevention is better than cure" and with the current style of living and that too in the present environmental scenario, exposure to metals is unavoidable and supplementation with natural antioxidants may be needed to combat metal induced toxicity (Valko *et al.*, 2006). It is of great concern that, the antioxidant used should be natural and efficacious in order to nullify the toxic manifestations of the metallic mixture. One such natural indole having a powerful antioxidant property, melatonin, was employed to check its protective ability against the multiple metal induced functional derailment of male reproductive organs and altering serum hormonal and clinical chemistry parameters.

## Material and Method:

For treatment, methodology employed and protocol refer material and methods section (Page no. 17).

# Results:

3-β HSD activity:

Animals exposed to the trimetallic mixture showed a marked decrease in the activity of 3-β HSD in testis in a duration dependent manner with maximal degree of inhibition at the longer duration time. Co-administration of melatonin showed significant protection against the TM mixture induced decrease in 3-β HSD activity in duration independent manner (Table: 8.1, Figure: 8.a and 8.b).

17-β HSD:

Duration dependent linear inhibition in 17-β HSD activity in testis was observed in TM mixture treated animals. The maximal inhibition of the testicular 17-β HSD activity was seen during the longer duration of exposure. Co-administration of melatonin showed marked protection against TM mixture induced decrement in testicular 17-β HSD activity (Table: 8.2, Figure: 8.2a and 8.2b).

Serum Testosterone:

TM mixture treatment exerted significant decrement in serum testosterone titre compared to the control animals. Interestingly, decrement in the serum testosterone titre was maximal with longer duration of exposure. Though melatonin exerted significant protection when simultaneously administered with Cd, the protective effect of melatonin was more pronouncedly seen at shorter duration of exposure. These changes in serum T level are represented in Table: 8.3, Figure: 8.3a and 8.3b.

Serum Estradiol:

TM mixture treatment resulted in lowered serum estradiol titre. This fall in $E_2$ was more pronounced at the shorter duration of exposure compared to longer duration of exposure. Simultaneous treatment with melatonin exerted significant protection against the TM mixture induced fall in serum estradiol titre (Table: 8.4, Figure: 8.4a and 8.4b).

Cauda Epididymal Sperm Count:

TM mixture treatment showed duration dependent linear decrease in cauda epididymal sperm count. The protective role of melatonin on cauda epididymal sperm number when co-administered with Cd was found to be better at longer duration of exposure (Table: 8.5, Figure: 8.5a and 8.5b).

Sperm motility:

Significant reduction in the number of motile sperm was observed in TM mixture administered animals. Minimum number of motile sperms was observed at the shorter duration exposure to TM. Co-administration of melatonin afforded marked protection

against the trimetallic mixture induced decline in sperm motility (Table: 8.6, Figure: 8.6a and 8.6b).

Sperm Abnormality:

The number of abnormal sperms increased with increasing duration of exposure to the trimetallic mixture with maximum number of abnormal sperms being recorded at 60 days of TM mixture exposure. Concurrent, simultaneous administration of melatonin showed significant protection against the TM mixture induced sperm abnormality with greater protective effect being noticeable with longer duration of treatment.

Histological observations:

Testis

Control testis sections revealed well formed seminiferous tubules with all stages of spermatogenesis represented. Testis of melatonin treated animals showed a quantitative reduction in the germ cell population of tubules with no qualitative effect on spermatogenesis. Testis of animals exposed to TM mixture show absence of sperms in the tubules with most of the meiotic and post meiotic germ cells undergoing degeneration. Spermatids and late spermatocytes could also be seen getting sloughed off from the germinal epithelium. Co-administrastion of melatonin along with the TM mixture showed protective effect with most of the tubules exhibiting spermatogenesis. The population of germ cells however was less dense and there was relatively more degeneration of germ cells compared to control testis.

Epididymis

Whereas melatonin showed cytoprotective effect on epididymal epithelium, the TM mixture showed inhibitory effects marked by shrinkage of caudal epididymal tubules

with hypotrophied epithelium. The lumens were mostly empty and contained degenerating germ cells. Coadministration of melatonin along with TM mixture showed significant protective effect as revealed by the well formed tubules which showed sperms in their lumen.

*In Vitro* Cell Viability

Whereas Melatonin showed a tendency for increasing cell viability, the TM mixture showed significantly increasing cytolethality over time. Simultaneous presence of melatonin afforded protection and improved cell viability to great extent. However, the percentage of cell viability was still significantly less than the control cells.

*In Vitro* Testosterone (T) Production:

Melatonin showed an inhibition in testosterone secretion which was more pronounced under hCG stimulation. TM mixture exposed cells showed pronouncedly decreased testosterone secretion under both basal and hCG stimulated conditions. The inhibition in testosterone secretion was found to be about 57% under both conditions. Cells exposed to TM mixture along with melatonin showed improvement in testosterone secretion markedly under hCG stimulation though still significantly lower than the testosterone secretion elicited by the control cells.

**Histological Observations**

Testis:

Testis of animals exposed to trimetallic mixture (Plate: 1, Fig. T1 and T2) show absence of sperms in the tubules with most of the meiotic and post-meiotic germ cell under degeneration. Spermatids and spermatocytes could all be seen sloughed off

from the germinal epithelium. Co-administration of melatonin (Plate: 1, Fig. T3 and T4) along with trimetallic mixture showed protective effect with most of the tubule exhibiting spermatogenesis. The population of germ cell however was thinner and there was relatively more degeneration of germ cells compared to control testis.

Epididymis

The trimetallic mixture (Plate: 2, Fig. E1 and E2) showed inhibition effects marked by shrinkage of cauda epididymis tubules and hypotrophid epithelium. The lumen was mostly empty and contained degenerative germ cells. Co-administration at melatonin (Plate: 2, Fig. E3 and E4) along with trimetallic mixture showed significant protective effect marked by well formed tubules which shows sperms in their lumen.

TUNEL Assay

Control testis (Plate: 3, Fig. T1 and T2) section showed milder TUNEL positiveness. Melatonin (Plate: 3, Fig. T3 and T4) treatment exerted relatively less TUNEL staining. Testis of TM mixture (Plate: 4, Fig. T1 and T2) treated rats showed high TUNEL positiveness compare to control. Co-administration of melatonin (Plate: 3, Fig. T3 and T4) along with TM mixture afforded apoptosis as seen in TM mixture alone treated TUNEL section revealed relatively less apoptosis.

Serum and tissue NO level:

In general melatonin administration increased both testicular and epididymal NO level and decreased serum NO level. TM mixture exerted marked decrement in serum NO and increment in tissue NO level. Melatonin co-administration further decreased serum NO level and showed a marked increased in epididymal NO level. (Table: 8.12).

# Plate: 1

Figure: $T_1$: Photomicrograph of testis treated with **trimetallic mixture** showing seminiferous tubules and Leydig cells (100X). Note the absence of mature germ cells in the tubules.

S: Seminiferous tubules; L: Leydig cells

Figure: $T_2$: Photomicrograph of testis treated with **trimetallic mixture** showing seminiferous tubules and Leydig cells (400X).Note the degenerating and detached germ cells and absence of mature germ cells

Figure: $T_3$ : Photomicrograph of testis treated with **trimetallic mixture** and Melatonin showing seminiferous tubules populated with germ cells (100X).

S: Seminiferous tubules; L: Leydig cells

Figure: $T_4$: Photomicrograph of testis treated with **trimetallic mixture** and Melatonin. Note the occurrence of progressive stages of germ cells though quantitatively less in number (400X).

**Plate: 1**

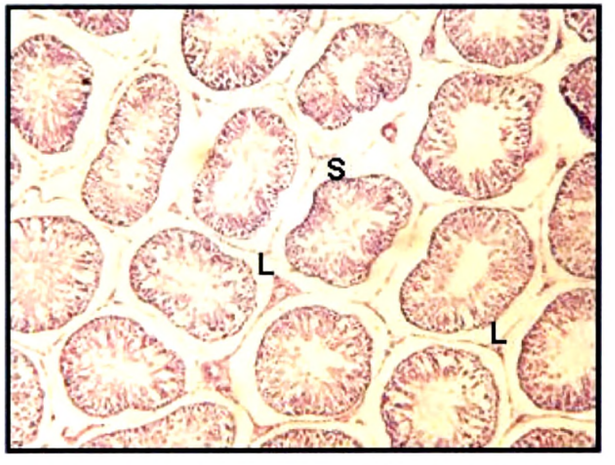


**Fig. T1: TM (100x)**

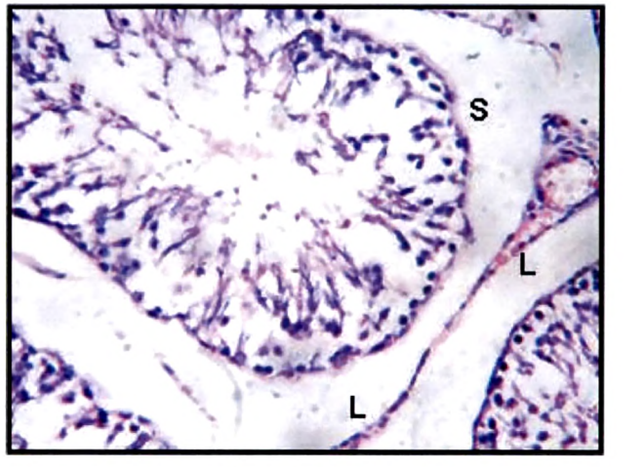


**Fig. T2: TM (400x)**

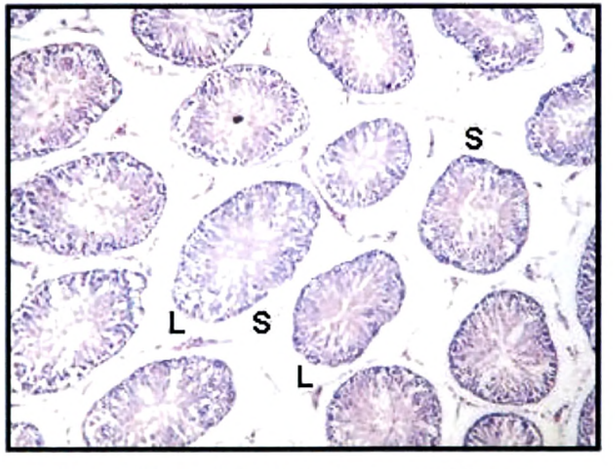


**Fig. T3: TM + Mel (100x)**

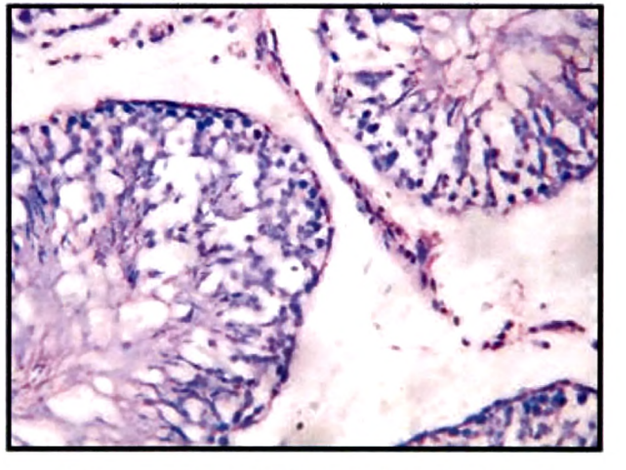

**Fig. T4: TM + Mel (400x)**

# Plate: 2

Figure: $E_1$: Photomicrograph of epididymis treated with **trimetallic mixture** showing shrunken cauda tubules (100X). note the absences of sperms in the lumen.

lu: Lumen

Figure: $E_2$: Photomicrograph of epididymis treated with **trimetallic mixture** (400X). Note the showing cauda tubules with hypertrophied epithelium and absences of sperms in the lumen

Figure: $E_3$: Photomicrograph of epididymis treated with **trimetallic mixture** and melatonin (100X) showing better formed cauda tubules and presences of sperms in many of the tubules

lu: Lumen; Sp: Sperms

Figure: $E_4$: Photomicrograph of epididymis treated with **trimetallic mixture** and melatonin (400X). Note the better appearance of tubule and presences of germ cells of the tubules

lu: Lumen; Sp: Sperms

**Plate: 2**

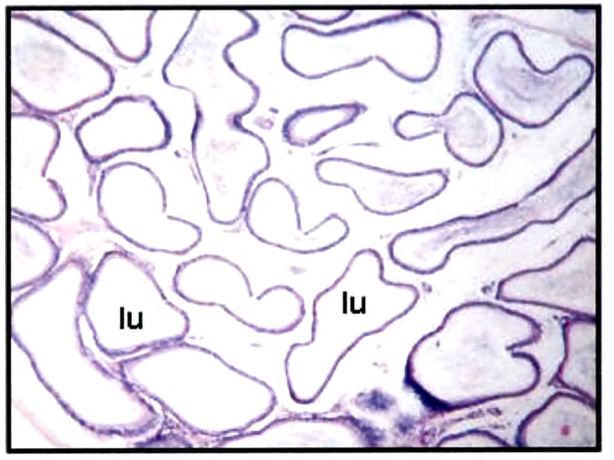


**Fig. E1: TM (100x)**

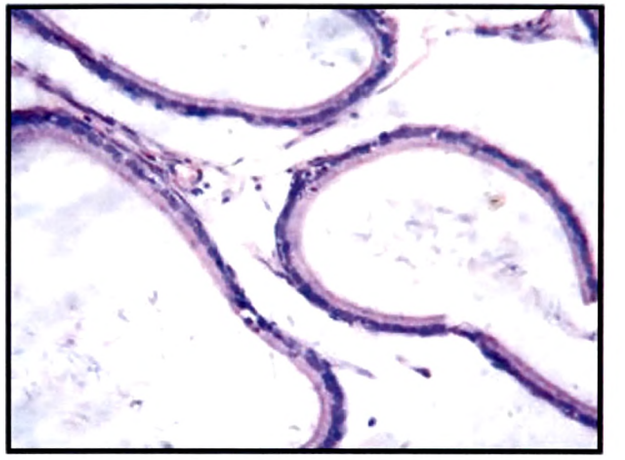

**Fig. E2: TM (400x)**

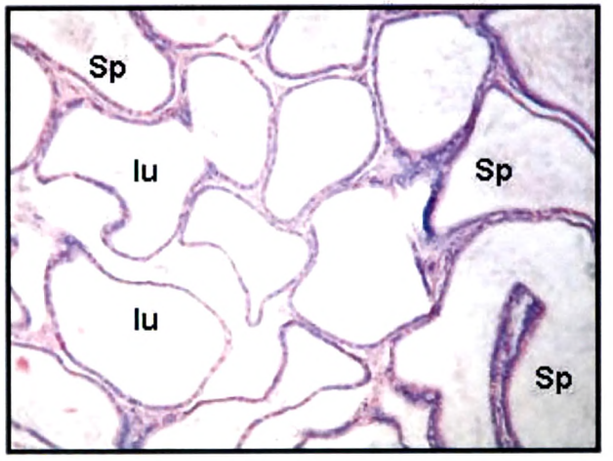


**Fig. E3: TM + Mel (100x)**

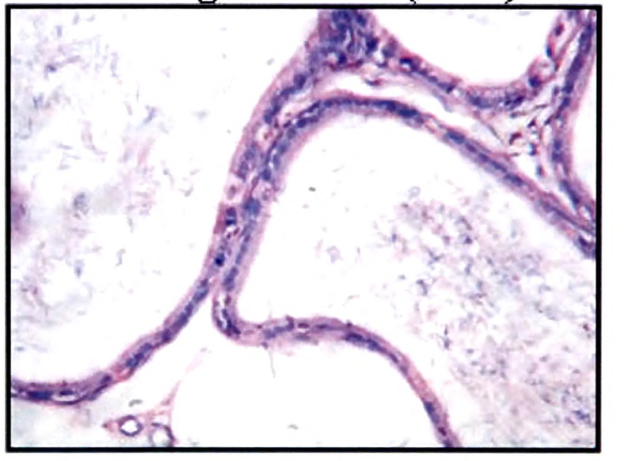

**Fig. E4: TM + Mel (400x)**

# Plate: 3

Figure: $T_1$: Control Testis subjected to qualitative TUNEL assay (400X). Note the near absence of TUNEL positive (Dark brown) cells.

Figure: $T_2$: Control Testis subjected to qualitative TUNEL assay (1000X).

Figure: $T_3$: Melatonin treated testis subjected to qualitative TUNEL assay (400X). Hardly few TUNEL positive cells (Dark brown) can be seen.

Figure: $T_4$: Melatonin treated testis subjected to qualitative TUNEL assay (1000X).

**Plate: 3**

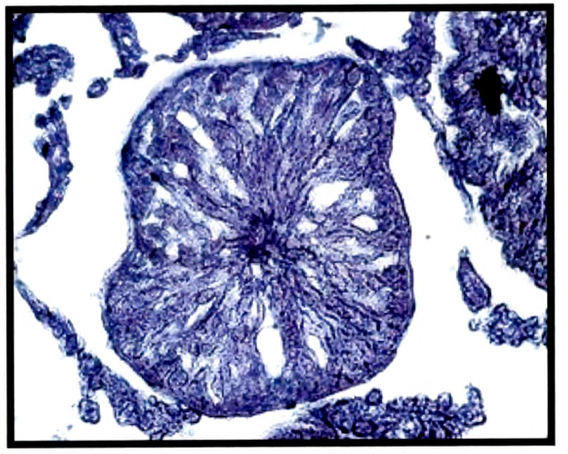

**Fig. T1: C (400x)**

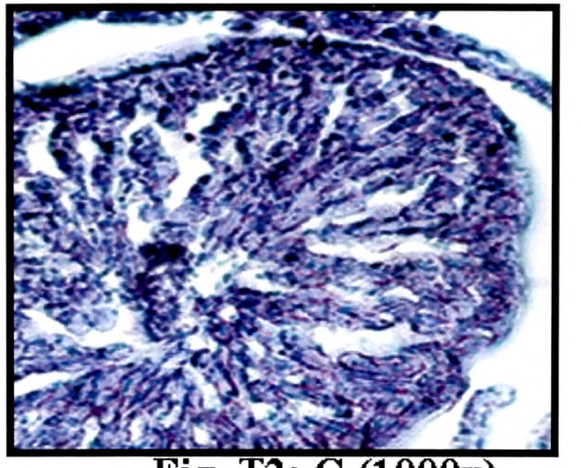

**Fig. T2: C (1000x)**

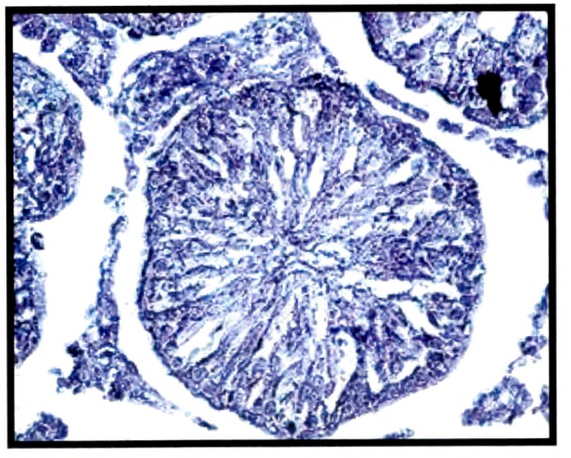

**Fig. T3: C + Mel (400x)**

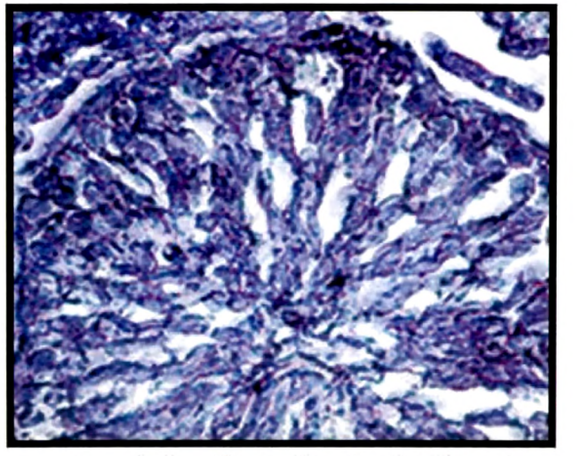

**Fig. T4: C + Mel (1000x)**

# Plate: 4

Figure: $T_1$: Trimetallic mixture exposed testis subjected to qualitative TUNEL assay (400X). Note the many TUNEL assay (Dark brown) positive cells.

Figure: $T_2$: Trimetallic mixture exposed testis subjected to qualitative TUNEL assay (1000X)

Figure: $T_3$: Trimetallic mixture and Melatonin treated testis subjected to qualitative TUNEL assay (400X). Note the presences of few TUNEL positive (Dark brown) cells.

Figure: $T_4$: Trimetallic mixture and Melatonin treated testis subjected to qualitative TUNEL assay (1000X)

Plate: 4

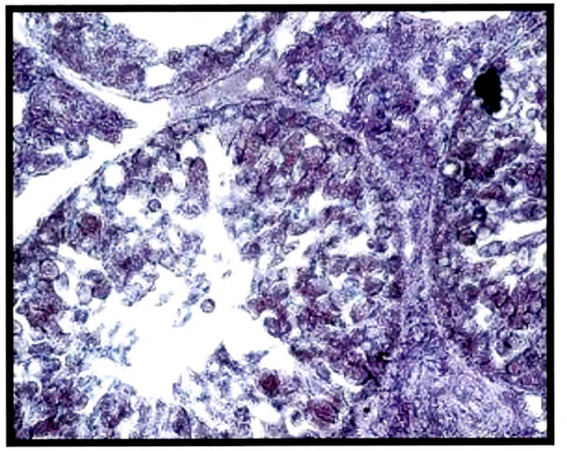

Fig. T1: TM (400x)

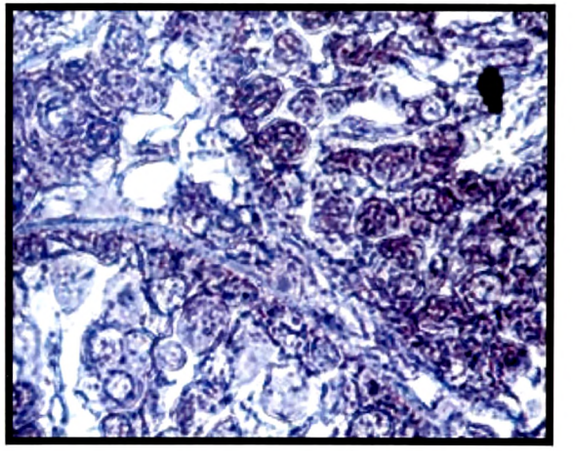

Fig. T2: TM (1000x)

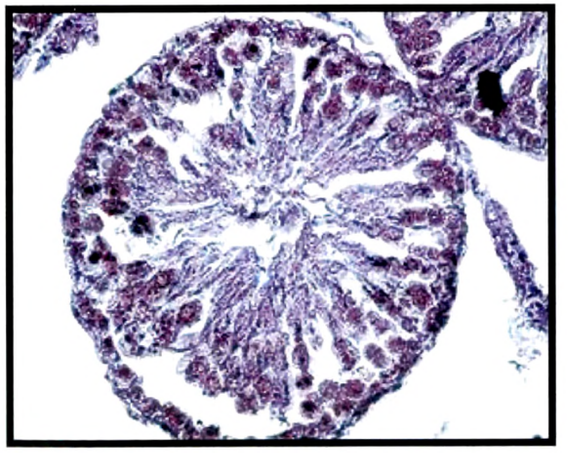

Fig. T3: TM + Mel (400x)

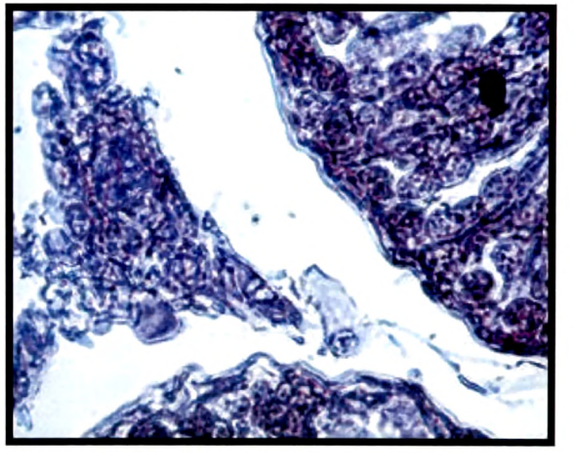

Fig. T4: TM + Mel (1000x)

**Figure 8.1a:** Percentage changes (decrease) in 3 – β HSD at 15, 30 and 60s of exposure to the TM mixture or the component metals individually.

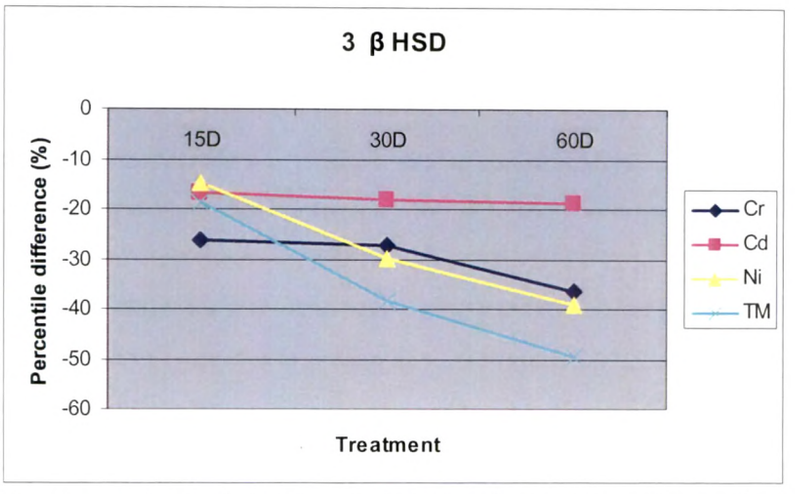


**Figure 8.1b:** Comparison of the temporal slope of percentage change in 3 – β HSD between TM mixture and component metals individually.

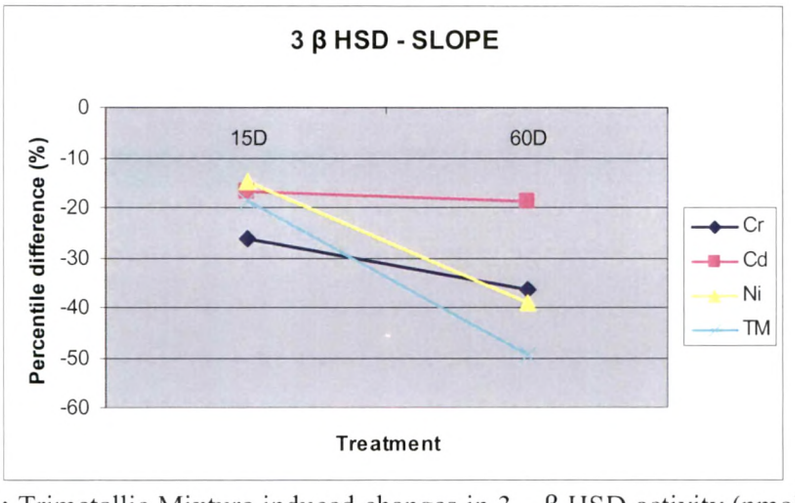


**Table 8.1:** Trimetallic Mixture induced changes in 3 – β HSD activity (nmol of NAD reduced/ min/ mg protein) in testis with or without Melatonin.

| Treatment | C | C + Mel | TM | TM + Mel |
|---|---|---|---|---|
| **15 Days** | 1.56 ± 0.02 | 1.49 ± 0.03 | 1.27 ± 0.13[b] | 1.22 ± 0.03 |
| **30 Days** | 1.54 ± 0.02 | 1.52 ± 0.02 | 0.95 ± 0.03[b] | 1.00 ± 0.05 |
| **60 Days** | 1.51 ± 0.02 | 1.43 ± 0.03 | 0.76 ± 0.02[b] | 1.19 ± 0.02[c] |

Values expressed as Mean ± SEM of 6 animals per group.
**a** $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
**c** $p<0.05$, compared with Trimetallic Mixture
C – Control; C + Mel – Control + Melatonin; TM – Trimetallic Mixture;
TM + Mel – Trimetallic Mixture + Melatonin

**Figure 8.2a:** Percentage changes (decrease) in 17 – β HSD at 15, 30 and 60s of exposure to the TM mixture or the component metals individually.

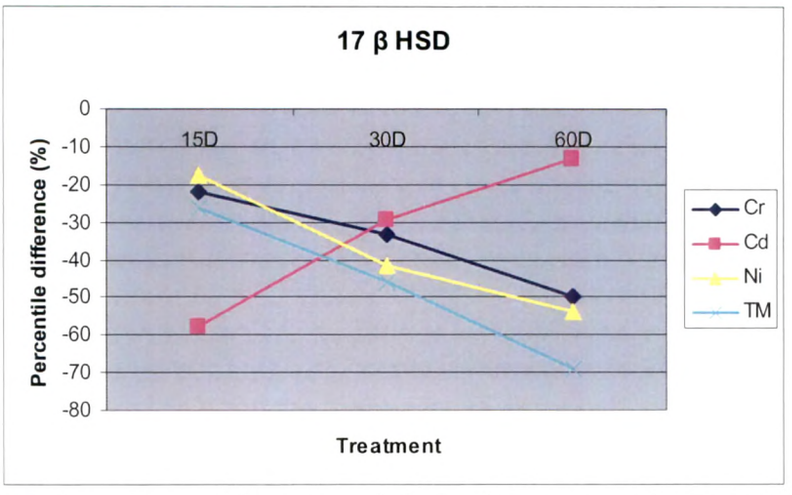


**Figure 8.2b:** Comparison of the temporal slope of percentage change in 17 – β HSD between TM mixture and component metals individually.

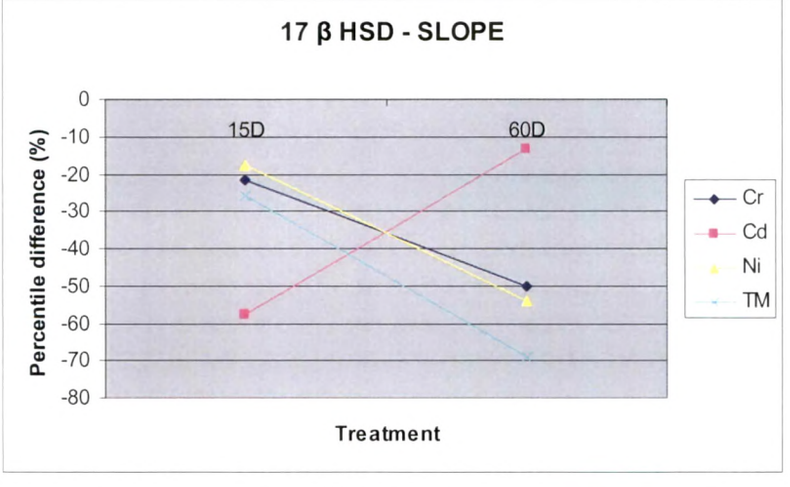


**Table 8.2:** Trimetallic Mixture induced changes in 17 – β HSD activity (nmol of NADP reduced/ min/ mg protein) in testis with or without Melatonin.

| Treatment | C | C + Mel | TM | TM + Mel |
|---|---|---|---|---|
| **15 Days** | 0.23 ± 0.01 | 0.22 ± 0.01 | 0.17 ± 0.01[b] | 0.19 ± 0.01 |
| **30 Days** | 0.24 ± 0.01 | 0.25 ± 0.01 | 0.13 ± 0.01[b] | 0.16 ± 0.01 |
| **60 Days** | 0.26 ± 0.01 | 0.24 ± 0.01 | 0.08 ± 0.01[b] | 0.17 ± 0.01[c] |

Values expressed as Mean ± SEM of 4 animals per group.
**a** $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
**c** $p<0.05$, compared with Trimetallic mixture
C – Control; C + Mel – Control + Melatonin; TM – Trimetallic Mixture;
TM + Mel – Trimetallic Mixture + Melatonin

**Figure 8.3a:** Percentage changes (decrease) in serum Testosterone(T) level at 15, 30 and 60s of exposure to the TM mixture or the component metals individually.

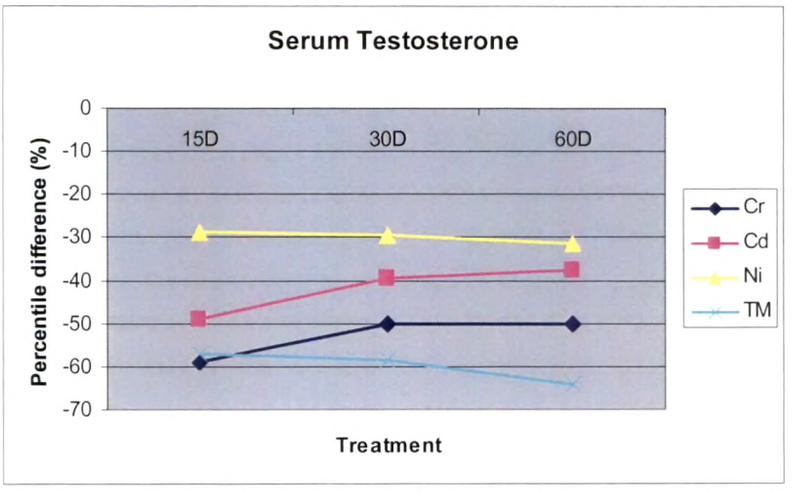


**Figure 8.3b:** Comparison of the temporal slope of percentage change in serum Testosterone(T) level between TM mixture and component metals individually.

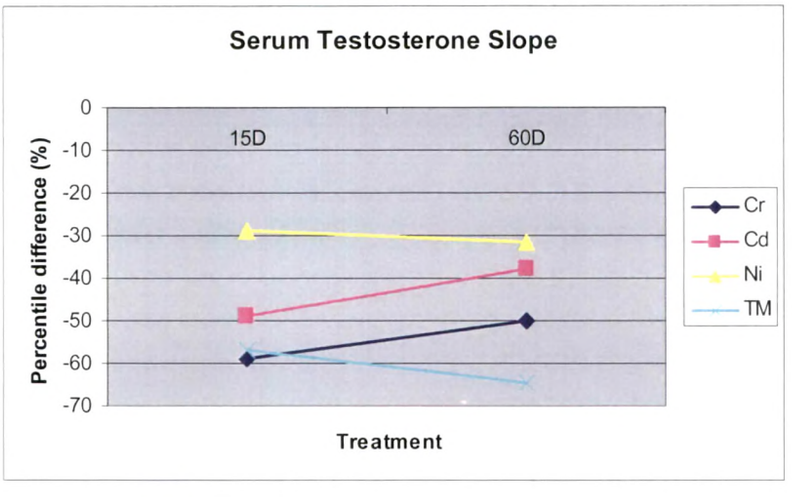


**Table 8.3:** Trimetallic Mixture induced changes in serum Testosterone(T) level (ng/ml of serum) with or without Melatonin.

| Treatment | C | C + Mel | TM | TM + Mel |
|---|---|---|---|---|
| **15 Days** | 3.59 ± 0.05 | 3.07 ± 0.05 | 1.55 ± 0.05[b] | 2.96 ± 0.04[c] |
| **30 Days** | 3.60 ± 0.18 | 3.04 ± 0.06[a] | 1.52 ± 0.17[b] | 2.23 ± 0.01[c] |
| **60 Days** | 3.88 ± 0.05 | 3.41 ± 0.06[a] | 1.38 ± 0.02[b] | 2.19 ± 0.03[c] |

Values expressed as Mean ± SEM of 4 animals per group.
**a** $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
**c** $p<0.05$, compared with Trimetallic mixture
C – Control; C + Mel – Control + Melatonin; TM – Trimetallic Mixture;
TM + Mel – Trimetallic Mixture + Melatonin

**Figure 8.4a:** Percentage changes (decrease) in serum Estradiol($E_2$) level at 15, 30 and 60s of exposure to the TM mixture or the component metals individually.

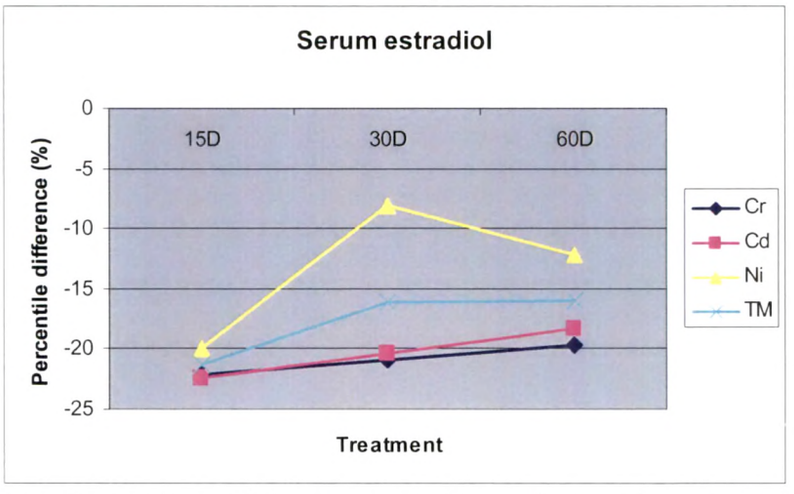


**Figure 8.4b:** Comparison of the temporal slope of percentage change in serum Estradiol($E_2$) level between TM mixture and component metals individually.

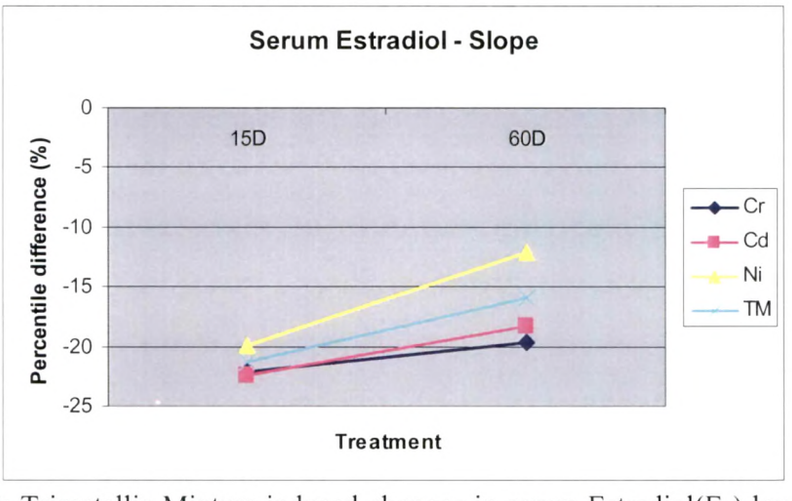


**Table 8.4:** Trimetallic Mixture induced changes in serum Estradiol($E_2$) level (pg/ ml of serum) with or without Melatonin.

| Treatment | C | C + Mel | TM | TM + Mel |
|---|---|---|---|---|
| **15 Days** | 34.5 ± 0.22 | 33.01 ± 0.12[a] | 27.17 ± 0.15[b] | 28.12 ± 0.34[c] |
| **30 Days** | 32.1 ± 0.13 | 30.83 ± 0.24 | 26.98 ± 1.93[b] | 27.54 ± 1.1 |
| **60 Days** | 34.01 ± 0.41 | 32.43 ± 0.73 | 28.59 ± 0.48[b] | 29.82 ± 0.28 |

Values expressed as Mean ± SEM of 4 animals per group.
**a** $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
**c** $p<0.05$, compared with Trimetallic mixture
C – Control; C + Mel – Control + Melatonin; TM – Trimetallic Mixture;
TM + Mel – Trimetallic Mixture + Melatonin

**Figure 8.5a:** Percentage changes (decrease) in epididymal sperm count at 15, 30 and 60s of exposure to the TM mixture or the component metals individually.

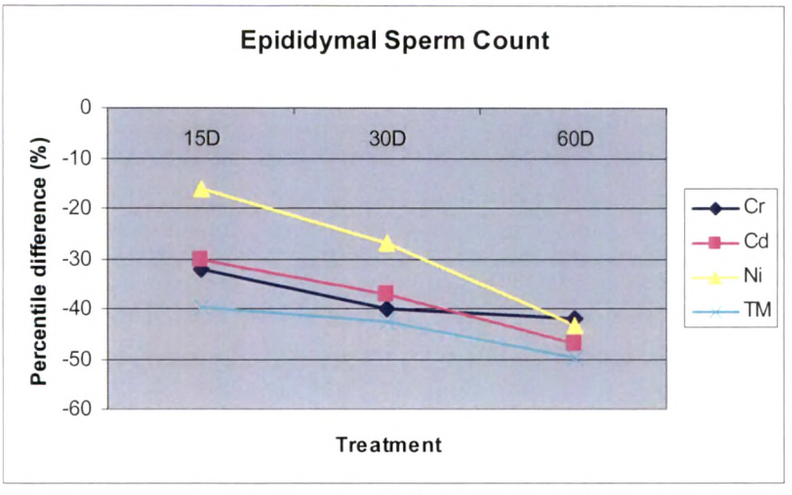


**Figure 8.5b:** Comparison of the temporal slope of percentage change in epididymal sperm count between TM mixture and component metals individually.

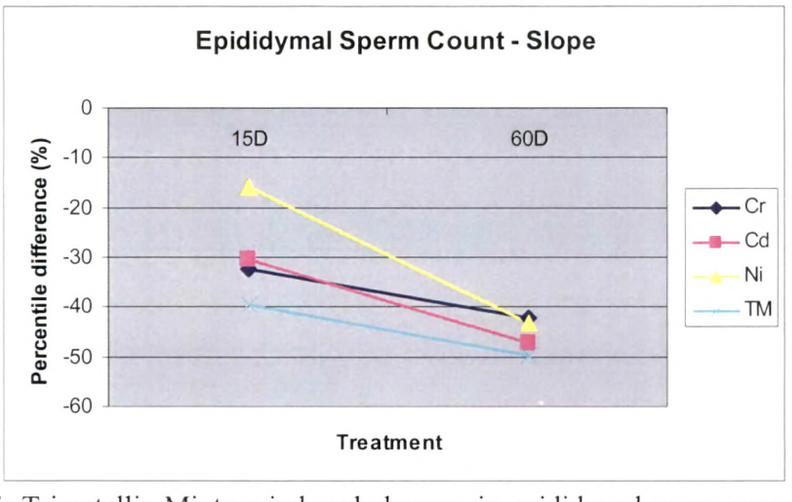


**Table 8.5:** Trimetallic Mixture induced changes in epididymal sperm count ($10^6$/ml) with or without Melatonin.

| Treatment | C | C + Mel | TM | TM + Mel |
|---|---|---|---|---|
| **15 Days** | 24.10 ± 0.07 | 23.14 ± 0.17 | 14.56 ± 0.50$^b$ | 15.6 ± 0.41 |
| **30 Days** | 22.44 ±0.13 | 22.10 ± 0.19 | 12.91 ± 0.40$^b$ | 13.71 ± 0.17 |
| **60 Days** | 24.08 ± 0.39 | 24.73 ± 0.19 | 12.10 ± 0.20$^b$ | 13.06 ± 0.06 |

Values expressed as Mean ± SEM of 4 animals per group.
**a** $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
**c** $p<0.05$, compared with Trimetallic mixture
C – Control; C + Mel – Control + Melatonin; TM – Trimetallic Mixture;
TM + Mel – Trimetallic Mixture + Melatonin

**Figure 8.6a:** Percentage changes (decrease) in epididymal sperm motility at 15, 30 and 60s of exposure to the TM mixture or the component metals individually.

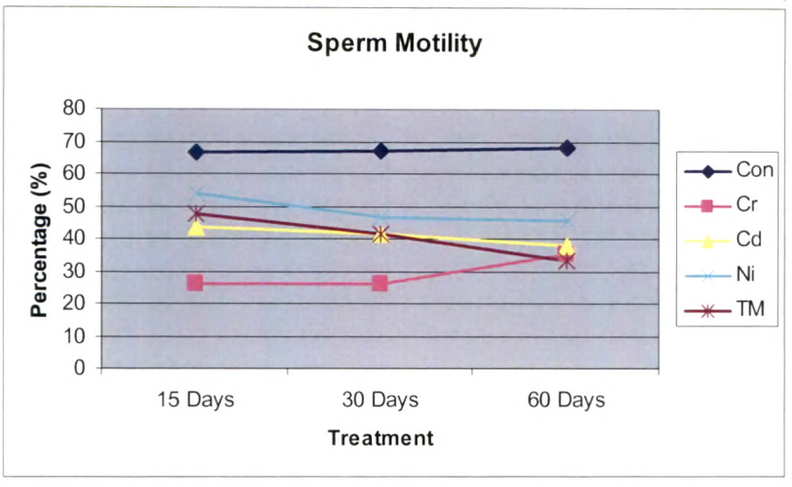


**Figure 8.6b:** Comparison of the temporal slope of percentage change in epididymal sperm motility between TM mixture and component metals individually.

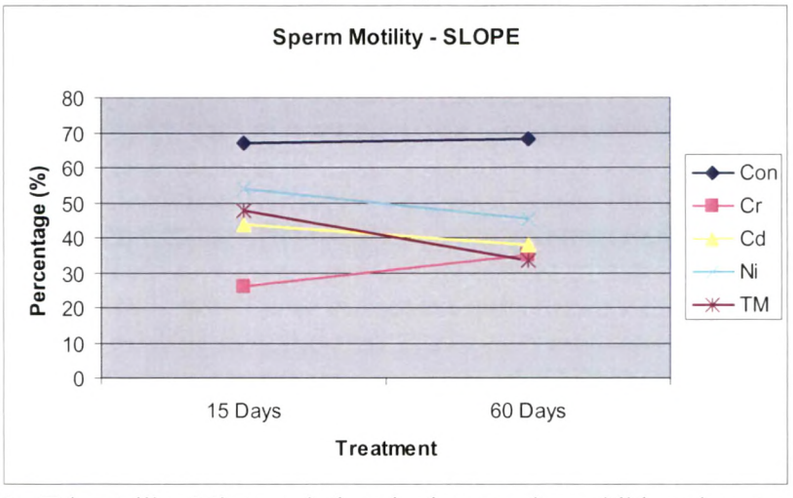


**Table 8.6:** Trimetallic Mixture induced changes in epididymal sperm motility (percentage) with or without Melatonin.

| Treatment | C | C + Mel | TM | TM + Mel |
|---|---|---|---|---|
| **15 Days** | 66.87 ± 0.12 | 66.92 ± 0.27 | 47.71 ± 0.26$^{b}$ | 52.75 ± 0.30$^{c}$ |
| **30 Days** | 67.16 ± 0.67 | 69.06 ± 0.46$^{a}$ | 41.68 ± 0.32$^{b}$ | 49.20 ± 0.25$^{c}$ |
| **60 Days** | 68.12 ± 0.50 | 67.44 ± 0.04 | 33.46 ± 0.14$^{b}$ | 37.16 ± 1.2$^{c}$ |

Values expressed as Mean ± SEM of 4 animals per group.
**a** $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
**c** $p<0.05$, compared with Trimetallic mixture
C – Control; C + Mel – Control + Melatonin; TM – Trimetallic Mixture;
TM + Mel – Trimetallic Mixture + Melatonin

**Figure 8.7a:** Percentage changes (decrease) in epididymal sperm abnormality at 15, 30 and 60s of exposure to the TM mixture or the component metals individually.

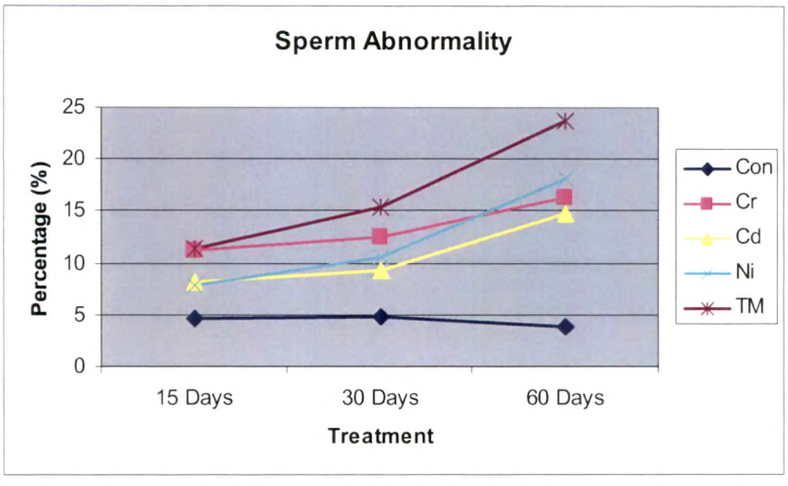


**Figure 8.7b:** Comparison of the temporal slope of percentage change in epididymal sperm abnormality between TM mixture and component metals individually.

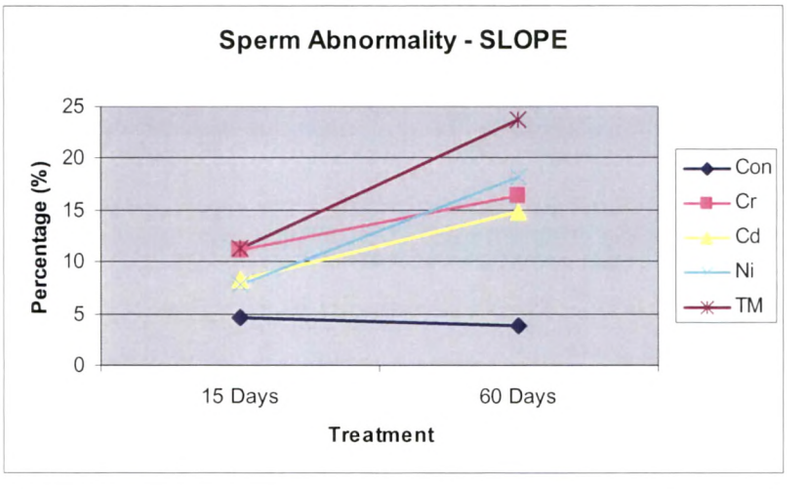


**Table 8.7:** Trimetallic Mixture induced changes in epididymal sperm abnormality (percentage) with or without Melatonin.

| Treatment | C | C + Mel | TM | TM + Mel |
|---|---|---|---|---|
| **15 Days** | 4.60 ± 0.2 | 3.14 ± 0.03[a] | 11.36 ± 0.44[b] | 10.19 ± 0.34[c] |
| **30 Days** | 4.80 ± 0.09 | 3.76 ± 0.10[a] | 15.37 ± 0.18[b] | 14.54 ± 0.25[c] |
| **60 Days** | 3.90 ± 0.2 | 3.05 ± 0.04 | 23.76 ± 0.45[b] | 15.91 ± 0.45[c] |

Values expressed as Mean ± SEM of 4 animals per group.
**a** $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
**c** $p<0.05$, compared with Trimetallic mixture
C – Control; C + Mel – Control + Melatonin; TM – Trimetallic Mixture;
TM + Mel – Trimetallic Mixture + Melatonin

**Table 8.8a:** Trimetallic Mixture induced percentage changes in chromium, cadmium and nickel content of testis with or without melatonin.

| Treatment | Cadmium | | | Chromium(VI) | | | Nickel | | |
|---|---|---|---|---|---|---|---|---|---|
| | 15 Days | 30 Days | 60 Days | 15 Days | 30 Days | 60 Days | 15 Days | 15 Days | 30 Days |
| **TM** | 28.95 | 31.11 | 55.74 | 35.00 | 15.38 | 23.81 | 10.88 | 10.83 | 24.38 |
| **TM + Mel** | -11.90 | 4.26 | 15.13 | 23.08 | 2.38 | 23.21 | 6.34 | 6.58 | 11.41 |

C – Control; C + Mel – Control + Melatonin; TM – Trimetallic Mixture;
TM + Mel – Trimetallic Mixture + Melatonin

**Table 8.8b:** Trimetallic Mixture induced percentage changes in chromium, cadmium and nickel content of epididymis with or without melatonin.

| Treatment | Cadmium | | | Chromium(VI) | | | Nickel | | |
|---|---|---|---|---|---|---|---|---|---|
| | 15 Days | 30 Days | 60 Days | 15 Days | 30 Days | 60 Days | 15 Days | 15 Days | 30 Days |
| **TM** | 31.12 | 12.39 | 28.76 | 18.49 | 22.00 | 23.72 | 3.58 | 11.99 | 21.28 |
| **TM + Mel** | 34.52 | 10.14 | 27.49 | 1.59 | -0.76 | 9.60 | -0.43 | 11.20 | 6.45 |

C – Control; C + Mel – Control + Melatonin; TM – Trimetallic Mixture;
TM + Mel – Trimetallic Mixture + Melatonin

**Table 8.9:** Trimetallic mixture induced changes in the serum titre of melatonin(pg/ ml) with or with out melatonin.

| Treatment | C | C + Mel | TM | TM + Mel |
|---|---|---|---|---|
| **15 Days** | 121.00 ± 0.36 | 140.00 ± 0.60[a] | 75.00 ± 0.45[b] | 102.00 ± 0.17[c] |
| **30 Days** | 112.00 ± 0.24 | 143.00 ± 0.17[a] | 60.00 ± 0.24[b] | 109.00 ± 0.61[c] |
| **60 Days** | 93.00 ± 0.05 | 126.00 ± 0.14[a] | 20.00 ± 0.34[b] | 87.00 ± 0.42[c] |

Values expressed as Mean ± SEM of 4 animals per group.
**a** $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
**c** $p<0.05$, compared with Trimetallic mixture
C – Control; C + Mel – Control + Melatonin; TM – Trimetallic Mixture;
TM + Mel – Trimetallic Mixture + Melatonin

**Table 8.10:** TM mixture induced changes in Leydig cell viability(in percentage) with or without melatonin.

| Treatment | Cell Viability | | | |
|---|---|---|---|---|
| | C | C + Mel | TM | TM + Mel |
| 3 Hours | 93.81 ± 0.06 | 94.10 ± 0.11 | 59.13 ± 0.24[b] | 69.42 ± 0.12[c] |
| 6 Hours | 93.42 ± 0.23 | 93.96 ± 0.24 | 51.70 ± 0.13[b] | 63.49 ± 0.46[c] |
| 12 Hours | 92.09 ± 0.07 | 94.33 ± 0.04 | 48.36 ± 0.22[b] | 60.13 ± 0.43 |

Values expressed as Mean ± SEM of 4 animals per group.
**a** $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
**c** $p<0.05$, compared with Trimetallic mixture
C – Control; C + Mel – Control + Melatonin; TM – Trimetallic Mixture;
TM + Mel – Trimetallic Mixture + Melatonin

**Table 8.11:** TM mixture induced changes in Testosterone (ng/ $10^6$ cells) secretion in isolated Leydig cell under basal and hCG stimulated condition with or without melatonin.

| Treatment | Testosterone Secretion (ng/ $10^6$ cells) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Basal | | | | hCG stimulated | | | |
| | C | C + Mel | TM | TM + Mel | C | C + M | TM | TM + Mel |
| 3 Hours | 4.87 ± 0.33 | 4.17 ± 0.16 | 2.08 ± 0.10[b] | 2.6 ± 0.06 | 7.58 ± 0.45 | 5.83 ± 0.04[a] | 3.46 ± 0.14[b] | 4.65 ± 0.02[c] |
| 6 Hours | 4.55 ± 0.16 | 4.06 ± 0.20 | 1.93 ± 0.23[b] | 2.49 ± 0.04[c] | 7.94 ± 0.04 | 5.93 ± 0.11 | 3.17 ± 0.24[b] | 4.26 ± 0.09[c] |

Values expressed as Mean ± SEM of 4 animals per group.
**a** $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
**c** $p<0.05$, compared with Trimetallic mixture
C – Control; C + Mel – Control + Melatonin; TM – Trimetallic Mixture;
TM + Mel – Trimetallic Mixture + Melatonin

**Table 8.12:** TM mixture induced changes in serum, testicular and epididymal NO level with or without melatonin

| Treatment Group | Serum NO | Testis NO | Epididymis NO |
|---|---|---|---|
| C | 7.23 ± 0.31 | 8.09 ± 0.36 | 13.54 ± 0.06 |
| C + Mel | 3.63 ± 0.61 | 11.46 ± 0.24 | 22.81 ± 0.13 |
| TM | 3.91 ± 0.25 | 9.11 ± 0.54 | 11.45 ± 0.02 |
| TM + Mel | 3.08 ± 0.30 | 9.24 ± 0.31 | 18.09 ± 0.32 |

Values expressed as Mean ± SEM of 4 animals per group.
**a** $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
**c** $p<0.05$, compared with Trimetallic mixture
C – Control; C + Mel – Control + Melatonin; TM – Trimetallic Mixture;
TM + Mel – Trimetallic Mixture + Melatonin

## Discussion:

Deciphering the effect of a chemical at the genetic level may well be characterized by toxicologists working in the field of molecular toxicology or even in the new field called toxicogenomics (Feron *et al.*, 2002; Thomas *et al.*, 2002). However, it is to be understood that chemicals can affect very many different physiological systems and, many of the body system also interact with each other (Carpenter *et al.*, 1998). This makes the interpretation of biologic relevance or health impacts of these changes very difficult Added to these is the possibility of interactions between component chemicals in a chemical mixture as well as their interactions with systems in the body, adding to a much higher level of complexity. In this context, an understanding of the effects of a chemical mixture of metals particularly at the environmentally relevant concentrations is meaningful in advancing a knowledge base on human health and environmental impact of mixtures. Interest and research on chemical mixtures have no doubt intensified during the last few years (Carpenter *et al.*, 2002; Feron *et al.*, 2002; Seed *et al.*, 1995). Complexity of interactions as reviewed above might give rise to opposing or confusing conclusions and as such, such results have been obtained (Monossen, 2005). Some recent studies on different life forms have shown toxicological interactions (antagonistic, additive or synergistic) of metals in a composite mixture (Lin *et al*, 2000; Franklin *et al.*, 2002; Otitogu, 2002; Spurgeon *et al.*, 2004). The current study has in this context attempted to assess the toxicological effects of a mixture of Cd, Cr and Ni. The degree of induction of oxidative stress and impact on steroidogenesis and spermatogenesis in the testis and functional competence of epididymis together with quantitative and qualitative effects on sperm have all been studied for each or these metals individually (Chapter1-3,5-7).

A trimetallic mixture of Cd, Cr and Ni given through drinking water at environmentally relevant concentrations have already shown heightened oxidative stress and disturbed functions of testis and epididymis (Chapter 4). In continuation, the present investigation shows significant inhibition of 3β and 17β HSD and lowered serum testosterone titre. Though, all the 3 metals individually were also effective in inhibiting the steroid dehydrogenase ( as well as lowering serum testosterone tire, the trimetallic mixture seems to project a greater toxicological effect essentially during the longer periods of exposure to the mixture. Compared to the effects of the 3 metals individually, the trimetallic mixture seems to exert an interactive synergistic effect (30 days) to an additive effect (60 days) on the degree of inhibition of 3β and 17β HSD. Apparently, prolonged long duration exposures to the mixture can develop an additive effect leading to a greater inhibition of steroid dehydrogenases. Similarly, synergistic and additive effect on increasing durations of exposure are for the serum testosterone titre, suggesting an additive effect of the three metals on Leydig cell steroidogenesis and testosterone secretion. In contrast, the serum tire of estrogen seems to be intermediate between Cd and Cr on one side and Ni on the other. This would suggest some degree of antagonism of Ni over Cd and Cr. Clearly, duration dependent complicated interactions between these metals can be envisaged. Some of the available literature on the interactive effects of metals are of antagonistic effect of Cr on Cd induced toxicity in rats( Stacey *et al.*, 1983), antagonistic effect on Cd induced alterations on Quail egg quality markers (Skalicke *et al.*, 2008), a combined effect of Cd and Ni on inducible cytochrome $P_{450}$ isozymes (Iscan *et al.*,1992), an additive effect of Cd and Ni on hepatic glutathione-S-transferase, but with differential effect renal enzyme (Iscan *et al.*, 1994), antagonistic effect of Cd in Cr induced apoptosis

(Schimade *et al.*,1998) and synergistic effect of Pb, Hg and Mn on central and peripheral nervous activity on rats(Papp *et al.*,2006). Except for such reports, there are no other studies available in the interactive effects of metals on testicular steroidogenesis and thereby precluding a meaningful discussion of the present observation.

The histopathological observation on testis and epididymis suggest marked inhibitory effects on testicular spermatogenesis and epididymal functions in relation to sperm morphology and maturation. Most of the seminiferous tubules show disturbed spermatogenesis with loss of sperms, spermatids and spermatocytes. The epididymal epithelium appears hypotrophied. Minimal number of (PVEC) characteristic epididymal basal cell modifications induced by Cr for entrapment of defective and or apoptotic spermatozoa (Chapter-5; Agnes and Akbarsha, 2001) are also observable with trimetallic exposure. These deleterious effects of trimetallic mixture on testis and epididymis are clearly reflected in the recorded significant decrease in epididymal are clearly reflected in the recorded significant decrease in epididymal sperm count and motility as well as on the increasing percentage of abnormal sperm of morphologically abnormal sperms. Though, there are no reports on metallic mixture induced effects on quantitative and qualitative aspects of epididymal sperms, the present study clearly indicate a synergistic effect of all the three metals in reducing the sperm count. The interactive effects of metals on epididymal functions can also be ganged from the comparative slope line graph of an antagonistic effect of Ni over Cr and Cd at short and medium durations of exposure and a synergistic effect of all the 3 metals on longer duration and an increasing additive effect from short to long duration exposure on the percentage increase of abnormal sperms. An endocrine correlation for decreased dysregulation in

spermatogenesis and epididymal functions may be sought in the herein recorded decreased testosterone / estradiol ratio of trimetallic mixture exposed animals, as both seminiferous tubules and epididymis are androgen sensitive tissues. The trimetallic mixture induced qualitative reduction on spermatogenesis can be related with an increased degree of germ cell apoptosis. The observed incidence of higher degree of germ cell apoptosis revealed by TUNEL positiveness provide evidence to that end. Further proof for the increased incidence of apoptosis is provided by the significantly increased Caspase-3 activity in the testis of TM mixture exposed animals. The possibility of occurrence of epididymal cell apoptosis can also be considered feasible as the caspase -3 activity in the epididymis is also increased significantly under tri metallic mixture exposure. Though there are no reports on epididymal caspase-3 activity or epididymal cells apoptosis, recent study (Aziz *et al.*, 2007) has shown a relation between sperm apoptosis induced before ejaculation (probably while within the epididymis) and sperm morphology attributes. One of the possible mechanisms of induction of apoptosis in testis and epididymis of trimetallic mixture exposed animals could be by way of activation of apoptotic pathway by the metal induced oxidative stress. Enough literature is available to substantiate the involvement of metal induced oxidative stress on cellular apoptosis (Turner and Lysiak, 2008). Such an induction of apoptosis in testis and epididymis is further substantiated by the recorded increase in tissues NO levels and in fact, increase in tissue NO levels have been correlated with cellular apoptosis. The reported existence of NO/ NO synthetase system in testis (Lee and Chen., 2004) further merits consideration in the context of oxidative stress induced apoptosis. As against the increase in tissue NO level the serum NO level is decreased and this may be viewed in the context of the role of metal especially Cd in inducing vascular hypertension. Since vasodilatation involve increased synthesis of

NO on vessel wall, the decreased serum NO can apparently be related with vasoconstriction, a characteristic Cd toxicity manifestation. It is interesting to note in this connection that, testis and epididymis of trimetallic exposed animals show relatively much higher accumulation of Cd than Cr and Ni. In this context number of studies have reported on oxidative stress induced testicular and epididymal dysfunctions (Marchlewicz *et al.*, 2004; Rajeshkumar *et al.*, 2002; Srilatha and Muralidhara., 2007; Kibeloma *et al.*, 2007; Kobayashi *et al.*, 2008).As in the case of studies involving individual metals (Chapters 5-7), even in the present study involving exposure to trimetallic mixture reveals the potent effect of Melatonin in preventing the various alterations affecting the testis and epididymis both structurally and functionally.

The observed histopathological observations suggesting severe lesion in spermatogenesis, apart from ROS and RNS mediated apoptosis could also be accounted for by the recorded significant decrement in serum testosterone level. This decrease in serum testosterone level could also result in lowered intratesticular testosterone level which could interfere with the process of spermatogenesis as, pendence of spermatogenesis on testosterone is an established dictum. Pertinently, the $T/E_2$ ratio is also much lower in the TM treated animals compared to animals exposed to individual metals (Chapters: 5-7).The detrimental $T/E_2$ ratio, contributed by the pronounced decrease in T secretion finds substantiation by the concurrently observed significant decrement in 3 β and 17 β HSD. The graphs on temporal slope for both the dehydrogenases and testosterone clearly indicate an additive inhibitory effect of all the three metals especially at the longer durations of exposure. Apparently, the inhibitory effect of Cd, Cr and Ni on steroid dehydrogenases and testosterone is additive when present together and has a much higher inhibitory potential compared

to that of the metals individually. These *in-vivo* observations and interpretations find justification from the *in-vitro* responses discussed below.

The *In Vitro* evaluation using isolated Leydig cells reveals greater degree of cytolethality compared to the degree seen with Cr, Cd and Ni individually (Chapters 5-7). In fact, the percentage of non-viable cells is the highest with exposure to the trimetallic mixture. Of the three metals, Cr, Cd and Ni, Cr had the greatest cytolethality effect (Chapter-5). Since the trimetallic mixture exerts a still higher degree of cytolethality, it is inferable that the three metals together in a mixture have a cumulative cytolethal effect. This is in contrast to the suppressive effect of Cd on Cr induced apoptosis in cultured Chinese hamster ovarian (CHO) cells (Shimada *et al.*, 1998). There are two possible explanations for this discrepancy. One possibility is that, the present study is on a trimetallic mixture with the presence of Ni as well and hence, Ni could have negated the suppressive effect of Cd on Cr induced cytolethality. Unless and until there is a strange as yet not revealed interaction between Ni, Cd and Cr, this appears to be a remote possibility as, Ni had shown the least cytolethality effect compared to Cr and Cd (Chapter- 6, 7). The alternative more plausible explanation could be that, the interactive effect of Cd and Cr is tissue or cell specific as, the current assay is on testicular Leydig cells while, the assay of Shimada *et al.* (1998) was on CHO cells. This is in keeping with the earlier reviewed differential interactions between metals but also the differential tissue responses and/or differential interactions between metals and metallic mixtures. Caution is therefore to be exercised while trying to assign a pan tissue or species response towards individual metals and metallic mixtures. Melatonin added simultaneous to the trimetallic mixtures exerts protective effect on the metal mixture induced cytolethality as can be inferred from the observed increased cell viability. As there is still

significant degree of cytolethality despite the presence of melatonin, it is easily deducible that the toxic effect of the trimetallic mixture is more potent and hence the inability of melatonin to offset the toxic effects of the metals totally. A closer scrutiny of the data on testosterone secretion from Leydig cells at 3 and 6 hours under basal and stimulated conditions clearly suggest an additive effect of all three metals in reducing testosterone secretion from Leydig cells as, the degree of inhibition in presence of the trimetallic mixture is greater than those for the individual metals alone (Chapter 5-7). Though Ni had the minimal inhibitory effect both Ni and trimetallic mixture show an identical degree of inhibition under basal and stimulated conditions while in the case of Cd and Cr, there had been an increased inhibitory effect under hCG stimulation compared to basal condition. So it is likely that while there is one single site of inhibition in the signal transduction pathway involving steroidogenesis for all the three metals, Cr and Cd when alone, or in the absence of Ni, probably acts at two different sites to decrease testosterone secretion further under hCG stimulation. Though, melatonin had an inhibitory effect on Leydig cell testosterone secretion, under both basal and hCG stimulated condition, melatonin shows a significant stimulatory effect in presence of TM mixture under both basal and hCG stimulated conditions. This observation, as was seen even in the case of individual metals, suggests an altered melatonin induced testosterone secretion dynamics. Melatonin apparently increases testosterone secretion from stressed Leydig cells. Even in this increased melatonin induced testosterone dynamics, the trimetallic mixture seems to have an additive effect as , the degree of increase in testosterone seen herein with trimetallic mixture is much greater than those seen with individual metal stressed Leydig cells (Chapter 5-7). Apparently, these intricacies of additive effect of Cr, Cd and Ni as well as the role of melatonin in altering the testosterone secretion dynamics

of metal stressed animals need to be studied at length to realize the molecular intricacies. It can be concluded that in general, the trimetallic mixture of Cr, Cd and Ni has both synergistic and additive effects on most of the parameters assessed herein as well as antagonistic effect of Ni on epididymal functions. It is also evident that melatonin has a remarkable protective effect and a combination supplementation therapy involving melatonin and other antioxidant vitamins could be more effective in potentiating the protective effect of melatonin on metal toxicity especially toward the toxicity induced by metallic mixtures.